REMARKS

The foregoing amendments are presented to place the application in compliance with the sequence rules under 37 CFR 1.821-1.825.

Applicants have submitted a Sequence Listing in both paper and computer readable form as required by 37 C.F.R. 1.821(c) and (e). Amendments directing its entry into the specification have also been incorporated herein. The content of the paper and computer readable copies are the same and no new matter has been added.

A copy of the Notice is also attached as required.

The specification has also been carefully reviewed and editorial changes have been effected. All of the changes are minor in nature and therefore do not require extensive discussion. Specifically, the specification headings have been added in conformance with U.S. practice. Also, a section entitled "Brief Description of the Drawings" has also been added in accordance with U.S. practice. The description presented in this new section is from the original drawings and thus does not represent new matter.

Further, the nucleotide and amino acid sequences disclosed throughout the specification and in Figures 1, 3-6, 8 and 9 have been identified and labeled in accordance with U.S. practice.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE**".

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

Respectfully submitted,

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Version with Markings to Show Changes Made

New genes containing a DNA sequence coding for hydroxynitrile lyase, recombinant proteins derived therefrom and having hydroxynitrile lyase activity, and use thereof

Background of the Invention

1. Field of the Invention

Biocatalytic processes have become very important for the chemical industry. In this connection, carrying out chemical reactions with the aid of biological catalysts is particularly interesting in those fields of application in which it is possible to utilize the frequently found enzyme property of preferably converting or forming in chemical reactions with chiral or prochiral components one of the two enantiomers.

Essential requirements for utilizing said advantageous properties of enzymes are the availability of said enzymes in industrially required amounts and a sufficiently high reactivity and also stability under the real conditions of an industrial process.

2. Description of the Related Art

Cyanohydrins are a particularly interesting class of chiral chemical compounds. Cyanohydrins are important, for example, in the synthesis of α-hydroxy acids, α-hydroxyketones, β-aminoalcohols which are used for producing biologically active substances, for example pharmaceutical active substances, vitamins or pyrethroid compounds.

Said cyanohydrins are prepared by addition of hydrocyanic acid to the carbonyl group of a ketone or aldehyde.

Industrial production of chiral compounds such as, for example, (S)-cyanohydrins was made possible by making use of the enzyme (S)-hydroxynitrile lyase from *Hevea brasiliensis* and is described, for example, in WO 97/03204, EP 0 951561 and EP 0 927 766.

However, there is a large variety of interesting chemical compounds for which the R enantiomers are important for industrial applications. Up until now, only processes which can be used only on the laboratory scale have been described for preparing a number of

products (e.g.: EP 0 276 375, EP 0 326 063, EP 0 547 655). In this connection, mainly enzyme preparations obtained from plants of the Rosaceae family, for example from the kernels of almonds *(Prunus amygdalus)*, were used.

Recently, *Prunus species* have become more and more important so that attempts were made to investigate said species in greater detail.

The specialist literature, for example Plant Physiology, April 1999, Vol 119, pp. 1535-1546, discloses that *Prunus species* can contain a plurality of R-HNL isoenzymes. These isoenzymes are expressed at different levels in various tissues of the plant. It was possible to identify in the plant *Prunus serotina* which is a close relative of *Prunus amygdalus* 5 different isoenzymes up until now and to sequence their genes. Only one *Prunus amygdalus* isoenzyme has been described up until now in Planta (1998) 206: 388-393, and this isoenzyme is most strongly expressed in the flower bud. A gene for said R-HNL isoenzyme has already been isolated and the cDNA has been sequenced.

However, no successful (functional) heterologous expression of such a gene has been reported in the specialist literature or patent literature.

Industrial applications on a large scale, too, have not been carried out up until now, the main reason being that enzyme preparations from almond kernels with hydroxynitrile lyase activity have not been available up until now in sufficient quantities and at justifiable costs.

Summary of the Invention

It was therefore an object of the invention to create a basis which can provide an R-hydroxynitrile lyase in amounts required for industrial applications.

This object was achieved by looking for a way of producing an enzyme corresponding to the R-HNL preparation of *Prunus amygdalus* by genetic engineering strategies with the aid of an appropriate recombinant microorganism strain. Such a recombinant enzyme with

primer combination of a primer 1 based on the DNA sequence of the 5'-region of the *mdl* genes from *Prunus serotina* and from *Prunus amygdalus* and/or a primer 2 based on the 3'-region of the DNA sequences of one of the hydroxynitrile lyase isoenzymes from *Prunus serotina* or from *Prunus amygdalus*, subsequent amplification with a DNA polymerase using a DNA from organisms, containing genes coding for hydroxynitrile lyase, as templates and cloning.

Thus it is possible, for example, to prepare gene-specific PCR primer based on sequence homology of the *Prunus amygdalus* MDL1 gene and of the *Prunus serotina mdl5* gene, and, as a result, a new gene, the *HNL5* gene, is obtained after amplification and cloning.

Insert Brief Description of the Drawings (see Amendments)

Description of the Preferred Embodiments

The *Prunus amygdalus HNL5* gene produced by PCR amplification, for example, has the nucleotide sequence depicted in figure 1, which is likewise a subject of the invention. The invention also relates to *HNL5* genes having a nucleotide sequence which is at least 80%, preferably 85%, identical to the sequence depicted in fig. 1.

The new *HNL5* gene differs from the published sequence of the *Prunus amygdalus* MDL 1 gene in 7 base pairs.

Furthermore it is possible, for example, to prepare gene-specific PCR primers based on sequence homology of the *Prunus serotina mdl1* gene, and, as a result, a new gene, the *HNL1* gene, is obtained after amplification and cloning.

The *HNL1* gene produced by PCR amplification, for example, has the nucleotide sequence depicted in figure 8, which is likewise a subject of the invention. The invention also relates to *HNL1* genes having a nucleotide sequence which is at least 80%, preferably 85%, identical to the sequence depicted in fig. 8.

Analogously, it is possible, according to the invention, to prepare further gene-specific PCR primers, for example based on the sequence of the *Prunus amygdalus* MDL1 gene and/or based on the sequence

preferably alkyl groups having 1 - 6 carbon atoms, and very preferably the cyanide group donor is acetonecyanohydrin.

The cyanide group donor may be prepared according to known methods. Cyanohydrins, in particular acetonecyanohydrin, may also be obtained commercially. Preference is given to using hydrocyanic acid (HCN), KCN, NaCN or acetonecyanohydrin as cyanide group donor, and particular preference is given to hydrocyanic acid.

In this connection, it is also possible to liberate hydrocyanic acid from one of its salts such as, for example, NaCN or KCN just prior to the reaction and to add it to the reaction mixture undissolved or in soluble form.

The conversion may be carried out in an organic, aqueous or 2-phase system or in emulsion. The aqueous system used is an aqueous solution containing the inventive HNL or a buffer solution. The examples thereof are Na citrate buffer, phosphate buffer, etc.

Organic diluents which may be used are aliphatic or aromatic hydrocarbons which are not or negligibly water-miscible and which are unhalogenated or halogenated, alcohols, ethers or esters or mixtures thereof. Preference is given to using methyl tert-butyl ether (MTBE), diisopropyl ether, dibutyl ether and ethyl acetate or a mixture thereof.

In this connection, the HNLs of the invention may be present either as such or immobilized in the organic diluent, but the conversion may also be carried out in a two-phase system or in emulsion using nonimmobilized HNL.

The present invention will now be described in more detail by the following Examples However, the following Examples are merely illustrative in nature. Other Examples within the scope of the claims are also possible. Thus, the following should not be construed to limit the spirit and scope of the claims.

Since it was known that a plurality of hydroxynitrile lyase isoenzymes whose sequences are highly homologous to one another can appear in *Prunus species* (Hu and Poulton, 1999), gene-specific PCR primers based on sequence homology of the *Prunus serotina mdl5* gene and the *Prunus amygdalus MDL1* gene (Suelves et al., 1998) were prepared:

Primer 1: 5'-CGGAATTCACAATATGGAGAAATCAACAATGTCAG-3' (SEQ ID No. 1)

Primer 2: 5'-CGGAATTCTTCACATGGACTCTTGAATATTATG-3' (SEQ ID No. 2)

The amplification was carried out in a 50 µl mixture with 1.2 U of "Hotstar" *Taq* DNA polymerase (Qiagen, Hilden, Germany), with 50 ng of genomic almond DNA as template, in each case 200 ng of primers 1 and 2, 5 µl of a dNTP (2 mM each) mix, all of these in 1x PCR buffer according to the "Hotstar Kit" manual (Qiagen, Hilden, Germany), starting with a denaturation step of 15 minutes at 95°C, followed by 30 cycles (1 min 95°C, 30 sec 64°C, 1 min 72°C) for amplification and a final incubation at 72°C for 5 min for preparation of complete products.

Said PCR produced a DNA fragment of approx. 2.16 kb in size (determined by analysis by means of agarose gel electrophoresis). This PCR product was purified by means of the "Qiaquick Kit" (Qiagen, Hilden, Germany) according to the enclosed manual and sequenced using the "Dye Deoxy Terminator Cycle Sequencing" kit (Applied Biosystems Inc., Forster City, CA, USA) according to the primer walking strategy starting from the two primers used for the PCR. The obtained DNA sequence of the PCR fragment of 2162 base pairs total length is depicted in figure 1.

Approx. 0.5 µg of the purified PCR product was cut with restriction endonuclease *Eco*RI and cloned into plasmid vector pBSSK(-) (Stratagene Cloning Systems, La Jolla, CA, USA) via the *Eco*RI cleavage site. The insert of a resultant recombinant molecule (the corresponding plasmid was denoted pBSPamHNL5g) was sequenced according to the method described above, and the sequence of the cloned fragment was 100% identical to

PamHnl5a1
5'-
GAAGATCTGAATTCCATGGAGAAATCAACAATGTCAGTTATACTATTTGTGTTGC
ATCTTCTTG-3'

PamHnl5a2
5'-
CTATTTGTGTTGCATCTTCTTGTTCTTCATCTTCAGTATTCAGAGGTTCACTCGCT
TGCCAATACTTC-3'

PamHnl5b
5'-
GTTCACTCGCTTGCCAATACTTCTGCTCATGATTTTAGCTACTTGAAGTTTGTGT
ACAACGCCACTG-3'

PamHnl5c
5'-GATGTATTGGAAGAGAAGAGGATCTTCTCTACT-3'

PamHnl5d
5'
GATCCTCTTCTCTTCCAATACATCAAATTTGTCAGCTATTGGAGTCATATATACG
G 3'

PamHnl5e
5'-
CAACCGGATTGACCTTTCTTGCAGGATTTGAAGGCCCACATACCTTCCTAACATC
AGATAGAAGCC-3'

PamHnl5f
5'-
GAAGATCTGGAATTCTTCACATGGACTCTTGAATATTATGAATAGCCTCCAACCG
GATTGACCTTTCTTGCAG-3'

Example 5

The preparation of a base fragment for constructing expression plasmids for heterologous expression of the *Prunus amygdalus HNL5* gene in bacteria and eukaryotes.

PamHnl5a1
5'-
GAAGATCTGAATTCCATGGAGAAATCAACAATGTCAGTTATACTATTTGTGTTGC
ATCTTCTTG-3' (SEQ ID No. 3)

PamHnl5a2
5'-
CTATTTGTGTTGCATCTTCTTGTTCTTCATCTTCAGTATTCAGAGGTTCACTCGCT
TGCCAATACTTC-3' (SEQ ID No. 4)

PamHnl5b
5'-
GTTCACTCGCTTGCCAATACTTCTGCTCATGATTTTAGCTACTTGAAGTTTGTGT
ACAACGCCACTG-3' (SEQ ID No. 5)

PamHnl5c
5'-GATGTATTGGAAGAGAAGAGGATCTTCTCTACT-3' (SEQ ID No. 6)

PamHnl5d
5'
GATCCTCTTCTCTTCCAATACATCAAATTTGTCAGCTATTGGAGTCATATATACG
G 3' (SEQ ID No. 7)

PamHnl5e
5'-
CAACCGGATTGACCTTTCTTGCAGGATTTGAAGGCCCACATACCTTCCTAACATC
AGATAGAAGCC-3' (SEQ ID No: 8)

PamHnl5f
5'-
GAAGATCTGGAATTCTTCACATGGACTCTTGAATATTATGAATAGCCTCCAACCG
GATTGACCTTTCTTGCAG-3' (SEQ ID No. 9)

Example 5

The preparation of a base fragment for constructing expression plasmids for heterologous expression of the *Prunus amygdalus HNL5* gene in bacteria and eukaryotes.

The aim of this experiment was to construct a plasmid from which a DNA fragment coding for *Prunus amygdalus HNL5* for incorporation into various expression vectors can be obtained by restriction endonuclease cleavage. In this connection, PCR amplification added suitable sequences to the ends of the *Prunus amygdalus HNL5* gene contained in pBSPamHNL5orf via appropriate primers.

The insert of plasmid pBSPamHNL5orf was amplified by means of PCR using the primers PCRHNL5-a and PCRHNL5-e (10 ng of DNA of plasmid pBSPamHNL5orf as template, 400 ng of primer PCRHNL5-a, 200 ng of primer PCRHNL5-e). The PCR reaction was carried out in 50 µl mixtures in 1x PCR buffer (Qiagen), containing 5 µl of the dNTP (2 mM each) mix and 1.2 U of "Hotstar" *Taq* DNA polymerase (Qiagen). The following program was run: 15 min 95°C, 30 cycles 1 min 95°C, 30 sec 68°C, 1.5 min 72°C, then finally 5 min 72°C for preparation of complete products).

After cutting with restriction endonuclease *Eco*RI, the DNA fragment obtained was cloned into vector pBSSK(-) (Stratagene, USA) and verified by sequencing. The resultant plasmid was called pBSPamHNL5ex.

Oligonucleotide primers:

PCRHNL5-a

5'-

TCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAAATAATTTTGTTTAACTTTA AGAAGGAGATATACATATGGAGAAATCAACAATGTCAGTTATACTATTTGTGTTG CATC-3' (SEQ ID No. 10)

PCRHNL5-e

5'-

CGAATTCGCCCTTTCGCATGCTCACATGGACTCTTGAATATTATGAATAGCCTC-3' (SEQ ID No. 11)

with *Eco*RI, via *Eco*RI cleavage sites simultaneously introduced at the ends of the DNA fragment. A clone having the correct orientation of the insert toward the *aox* promoter of plasmid pHILD2 was verified by sequencing the transition regions from vector to insert and preserved. The plasmid constructed in this way was denoted pHILDPamHNL5gox.

Not1-linearized DNA of plasmid pHILDPamHNL5gox was transformed into the strain *Pichia pastoris* GS115 and also into the protease-deficient strain *Pichia pastoris* SMD1168. From each mixture, several histidine-prototrophic clones were cultured in shaker flasks and HNL activity was determined in the cultured supernatant (standard assay). These experiments were carried out analogously, as described in example 7. It was possible to find in the culture supernatant of some clones HNL activity and thus to state that the signal sequence of the *Aspergillus niger gox* gene is capable of directing the heterologous HNL5 protein, when expressed in *Pichia pastoris*, into the secretory pathway.

Oligonucleotide primers used

GLUCOX1
5'-
CACGAATTCATCATGCAGACTCTCCTTGTGAGCTCGCTTGTGGTCTCCCTCGCT
GCGGCCCTGCCACACTAC-3' (SEQ ID No. 12)

GLUCOX2
5'-
TGCGGCCCTGCCACACTACATCAGGAGCAATGGCATTGAAGCCTACAACGCCA
CTGATACAAGCTCGGAAGGATC-3' (SEQ ID No. 13)

GLUCOXCT
5'-GAATTCGCATGCGGCCGCTCACTGCATTGACCTTTCTTGCAGGATTTGAAG-3' (SEQ ID No. 14)

The nucleic acid sequence of the DNA fragment for a secretory hybrid protein (PamHNL5xGOX) with HNL activity is depicted in figure 4, and the amino acid sequence derived therefrom is represented in figure 5.

Sample 6: 2.4 U of *Pam*HNL5 were incubated with 50 mU of endoglycosidase H in 20 mM phosphate buffer without denaturation at 37°C for 12 hours.

Sample 7: 2.4 U of *Pam*HNL5 were incubated with 50 mU of endoglycosidase H in 20 mM phosphate buffer, 0.2% SDS, 0.4% mercaptoethanol at 37°C for 12 hours.

After treatment with the glycosidases, the samples were separated on a 12 percent strength SDS polyacrylamide gel and stained with Coomassie Blue.

These results (see figure 7) show that a large part of the oligosaccharides bound to *Pam*HNL5 can be removed by endoglycosidase H even without denaturation of the *Pam*HNL5 protein.

Cleaving off the oligosaccharides leads from a protein smear visible around sizes of from 70 to over 100 kDa to a sharp band at about 60 kDa, corresponding to the calculated molecular weight of a nonglycosylated *Pam*HNL5 protein.

A comparable protein band is not present in the Roche preparation or present only to a negligible extent. In addition, it is impossible to see a significant difference between an untreated protein preparation and a preparation treated with endoglycosidase F. From this finding, it can definitely be stated that the recombinant *Pam*HNL5 enzyme is completely different from the enzyme material obtained from almonds.

Example 12:

Cloning of a genomic DNA fragment having the coding region of the *Prunus amygdalus HNL*1 gene.

A genomic DNA fragment having the coding region of the *Prunus amygdalus HNL*1 gene was amplified from genomic almond DNA (preparation, see example 1) with the aid of a PCR using primers mandlp2f (5'-ACTACGAATTCGACCATGGAGAAATCAAC-3') and ecpamHNL1e (Seq ID No. 15)

(5'-CAGAATTCGCCCTTGTGCATGCATCGATTAAAGAACCAAGGATGCTGCTGA C-3') (SEQ ID No. 16)

The amplification was carried out in 50 µl reactions with 1.2 units of "Hotstar" DNA polymerase (Qiagen GmbH, Hilden, Germany), in each case 10 pmol of the two primers, 2 µl of a dNTP mix (5 mM each) and 100 ng of genomic almond DNA in standard PCR buffer (Qiagen GmbH, Hilden, Germany). The following PCR program was used: 15 min 95°C, then 10 cycles of 1 min at 94°C, 1 min 45°C and 1 min 20 sec at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 64°C and 1 min 20 sec at 72°C and a final extension step at 72°C for 5 min.

Analysis of the DNA obtained showed that this PCR produced a plurality of DNA fragments of different sizes. Amplified DNA was separated in a preparative agarose gel. DNA from a band of the size to be expected for the *HNL1* gene of approx. 2.1 kb was isolated from said agarose gel (Qiaquick Gel Extraction Kit, Qiagen GmbH, Hilden, Germany). The DNA obtained, after digest with restriction endonuclease *Eco*RI, was cloned into cloning vector pBSSK(-) (Stratagene Cloning Systems, La Jolla, CA, USA) via the *Eco*RI cleavage site. 5 clones with appropriate inserts were isolated and the inserts were sequenced by means of the primer walking strategy. A clone corresponding to the consensus sequence obtained in this way was selected and the contained plasmid was denoted pSKpamHNL1_5_3.

The DNA sequence of the *Prunus amygdalus HNL1* gene was verified and finally determined by amplifying another genomic DNA fragment using primers mandlp3f (5'-ACTACGAATTCGACCATGGAGAAATCAACAATG-3') (SEQ ID No 17) and pamHNL1end (5'-ATGCTGCTGACTTGAGGGAATC-3') (SEQ ID No. 18). The amplification was carried out in 50 µl reactions with 2.5 units of "Hotstar" DNA polymerase (Qiagen GmbH, Hilden, Germany), in each case 10 pmol of the two primers, 2 µl of a dNTP mix (5 mM each) and 50 ng of genomic almond DNA in standard PCR buffer (Qiagen GmbH, Hilden, Germany). The following PCR program was used:

~~Patent Claims:~~

What is claimed is:

1. A new gene containing a DNA sequence coding for hydroxynitrile lyase, which gene can be prepared via a primer combination based on the DNA sequence of the 5'-region of the *mdl* genes from *Prunus serotina* and from *Prunus amygdalus* and/or a primer 2 based on the 3'-region of the DNA sequences of one of the hydroxynitrile lyase isoenzymes from *Prunus serotina* or from *Prunus amygdalus*, subsequent amplification with a DNA polymerase using a DNA from organisms, containing genes coding for hydroxynitrile lyase, as templates and cloning.

2. The new gene as claimed in claim 1, which can be prepared from primers based on the sequences of the *Prunus amygdalus* MDL1 gene and of one of the *Prunus serotina mdl* genes, and the subsequent amplification and cloning.

3. The new gene as claimed in claim 1, which can be prepared from primers based on the sequences of the *Prunus serotina mdl5* gene and of the *Prunus amygdalus* MDL1 gene, subsequent amplification and cloning, which gene has the nucleotide sequence depicted in figure 1 or is at least 80% identical thereto.

4. The new gene as claimed in claim 1, which can be prepared from primers based on the sequence of the *Prunus serotina mdl1* gene, subsequent amplification and cloning, which has the nucleotide sequence depicted in figure 8 or is at least 80% identical thereto.

5. The new gene as claimed in claim 1,which has the nucleotide sequence depicted in figure 1 from nucleotide 13 until nucleotide 2151 continuously or without the intron regions from nucleotide 116 until 257, 918 until 1120 and 1962 until 2077.

6. The new gene as claimed in claim 1,which has the nucleotide sequence depicted in figure 8 from nucleotide 1 until nucleotide 2083 continuously or without the intron regions from nucleotide 104 until 249, 907 until 1047 and 1889 until 1993.

Abstract OF THE DISCLOSURE

New genes containing a DNA sequence coding for hydroxynitrile lyase, which genes can be prepared via a primer combination based on the DNA sequence ~~of~~ of the 5′-region of the *mdl* genes from *Prunus serotina* and from *Prunus amygdalus* and/or a primer 2 based on the 3′-region of the DNA sequences of one of the hydroxynitrile lyase isoenzymes from *Prunus serotina* or from *Prunus amygdalus*, subsequent amplification with a DNA polymerase using a DNA from organisms, containing genes coding for hydroxynitrile lyase, as templates and cloning, and also recombinant proteins which can be prepared in suitable host cells by heterologous expression of the DNA sequence of said *HNL* genes, and proteins and fusion proteins derived therefrom and use of said proteins for preparing (R)-or (S)-cyanohydrins.